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Biodegradation of bisphenol A and its metabolic intermediates by activated sludge: Stoichiometry and kinetics analysis

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ABSTRACT

Bisphenol A (BPA) has been described as a estrogenic compound. However, it is used in the production of polycarbonate, epoxy resins and other plastics. Mixed microbial cultures, such as activated sludge, have been proposed as a feasible methodology to achieve the decontamination of wastewater containing BPA. Several studies on the biodegradation of BPA have demonstrated the presence of 4-hydroxyacetophenone (4HAP), 4-hydroxybenzaldehyde (4HB) and 4-hydroxybenzoic acid (A4HB) as the main metabolic intermediates. The objective of the present work was to study the kinetics and stoichiometric characteristics of the aerobic degradation of BPA and its metabolic intermediates by activated sludge. The effect of the sludge age (SA), and the concentration of each compound was analyzed. The biodegradation of the compounds was analyzed by an open respirometer. The kinetics of the biodegradation of all the tested compounds was influenced by the SA, the acclimation process, and the concentration of the tested compound. The stoichiometry of the reactions was not affected by the studied conditions. The oxidation coefficient ($Y_{O/S}$) was 10.0 ± 0.5, 5.2 ± 0.2, 3.7 ± 0.2, and 3.0 ± 0.1 $molO_2$ $mols^{-1}$ for BPA, 4HAP, 4HB, and 4HBA, respectively. Based on the obtained $Y_{O/S}$ values, the aerobic biodegradation pathway of the tested compounds by BPA-acclimated activated sludge is discussed.

1. Introduction

As a result of its wide usage as an intermediate in the production of polycarbonate plastics and epoxy resins, bisphenol A (BPA) is commonly detected in wastewaters (Melcer and Klecka, 2011). Unfortunately, BPA had adverse effects as an endocrine disruptor, and became a major concern (Omoike et al., 2013). Considerable data indicate that exposure of humans to BPA is associated with increased risk for cardiovascular disease, miscarriages, decreased birth weight at term, breast and prostate cancer, reproductive and sexual dysfunctions, altered immune system activity, metabolic problems and diabetes in adults, and cognitive and behavioral development in young children (Braun et al., 2009, 2011; Rees-Clayton et al., 2011; Lang et al., 2008; Li et al., 2009; Miao et al., 2011; Sugiura-Ogasawara et al., 2005). These findings are entirely consistent with parallel studies that demonstrate reproductive, metabolic and neuro-developmental problems in animals exposed to environmentally relevant levels of BPA (Salian et al., 2009; Wei et al., 2011; Wolstenholme et al., 2011).

Utilizing the metabolic potentials of microorganisms to mineralize pollutants is a safer and economic alternative to the widely used physico-chemical processes (Omoike et al., 2013). Microbial metabolism, pure and mixed cultures, have been described as a potential technology for detoxification of BPA-containing wastewaters (Sakai et al., 2007; Zhang et al., 2007; Kabiersch et al., 2011). Different pathways for the biodegradation of BPA has been described (Gao et al., 2010). In general, the most frequently detected metabolic products include 4-hidroxyacetophenone (4HAP), 4-hydroxybenzaldehyde (4HB) and the 4-hydroxybenzoic acid (4HBA) (Spivack et al., 1994; Sasaki et al., 2005; Sakai et al., 2007; Zhang et al., 2007). To assess the impact of the discharge of BPA to the environment, the biodegradability of BPA degradation products should also be considered. In this sense, the objective of the present work was to evaluate the kinetics and stoichiometry of

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the aerobic degradation of BPA and its metabolic intermediates 4HAP, 4HB, and 4HBA by activated sludge. The effect of sludge age (SA) and substrate concentration on the kinetic and stoichiometric parameters was also analyzed.

2. Materials and methods

2.1. Chemicals and reagents

Bisphenol A (BPA), 4-hydroxyacetophenone (4HAP), 4-hydroxybenzoic acid (4HBA), and 4-hydroxybenzaldehyde (4HB) (≥99%) were from Sigma–Aldrich. Dehydrated Cheese whey was from Food S.A. (Villa Maipú, Argentina). All inorganic salts were commercial products of reagent grade from Anedra (San Fernando, Argentina).

2.2. Activated sludge

Activated sludge used in this study was cultured in an aerobic laboratory-scale (4.5 l) activated sludge reactor with partial biomass recycle. The sludge age (SA) was maintained at 30 or 45 d (SA_{30} and SA_{45}, respectively) by daily wasting of the mixed liquor directly from the reactor. In both cases the hydraulic retention time was 48 h. The reactor was fed with the following synthetic wastewater: dehydrated cheese whey (CW) 1.5 g (1500 mg COD l^{-1}), $(NH_4)_2SO_4$ 0.5 g, and $NaHCO_3$ 1.03 g, all dissolved in 1 l of tap water (Ferro Orozco et al., 2010). Operating temperature was 20 ± 2 °C. Aeration was provided by an air pump; air was pumped near the bottom of the reactor and the dissolved oxygen (DO) concentration was above 4 mg l^{-1}. Under steady-state conditions pH was 7.5 ± 0.4, COD of the effluent ranged from 30 to 80 mg COD l^{-1}, and total suspended solids (TSS) concentration ranged from 3700 to 4500 mgTSS l^{-1}.

2.3. Acclimation of activated sludge to bisphenol A

Consecutive batch assays were performed to obtain activated sludge acclimated to BPA (Ferro Orozco et al., 2013). The inoculum for the first batch was obtained from the aerobic laboratory-scale activated sludge reactor described in Section 2.2. Then, the inoculum for the successive batch assays was obtained from the previous one. Before starting each batch, the biomass was harvested by sedimentation and washed with phosphate buffer (KH_2PO_4 2 g l^{-1}, K_2HPO_4 0.5 g l^{-1}, pH = 7.0). The washed biomass was re-suspended in the fresh culture medium to serve as inoculum for the next batch assay. The composition of the culture medium used to acclimate the biomass to BPA was the following: BPA (40 mg l^{-1}), $(NH_4)_2SO_4$ 220 mgN l^{-1}, KH_2PO_4 2 g l^{-1}, K_2HPO_4 0.5 g l^{-1}, and 1 ml l^{-1} of micronutrient solutions M1 and M2, respectively. The composition of M1 was (g/100 ml): $FeSO_4.7H_2O$ 1.5, $ZnSO_4.7H_2O$ 0.5, $MnSO_4.H_2O$ 0.3, $CuSO_4.5H_2O$ 0.075, $CoCl_2.6H_2O$ 0.015, and citric acid 0.6. M2 solution contained the following (g/100 ml): $(NH_4)_6Mo_7O_{24}.4H_2O$ 0.05, H_3BO_3 0.01, KI 0.01. Initial pH of the culture medium was adjusted to 7.0 ± 0.2 using NaOH or H_2SO_4 (1 N) solutions. For each batch, samples were taken at different times to determine soluble total organic carbon (TOCs) and BPA concentrations. Total suspended solids (TSS) were used as a measure for the biomass concentration (X); duplicate TSS was determined at least at the beginning and at the end of each batch. The specific BPA degradation rate (q_{BPA}) was used to evaluate the acclimation degree of the activated sludge during the acclimation process (Ferro Orozco et al., 2013).

2.4. Biodegradation assays: the open respirometer

The aerobic biodegradation of bisphenol A (BPA), 4-hydroxyacetophenone (4HAP), 4-hydroxybenzaldehyde (4HB), and 4-hydroxybenzoic acid (4HBA) by BPA-acclimated activated sludge was assessed using an open flowing gas/static liquid respirometer. Before performing the assays, activated sludge samples (500 ml) were washed three times using a phosphate buffer (KH_2PO_4 2 g l^{-1}, K_2HPO_4 0.5 g l^{-1}, pH = 7.0) and resuspended in the same buffer. Then, 500 ml of the washed activated sludge were poured into the open respirometer. Agitation was provided by a magnetic stir-bar; the respirometer was aerated continuously by an air pump. Air was set to a stable flow rate (1.0 l min^{-1}) using a high precision rotameter (Bruno Schilling model MB 60V, Argentina). Operation temperature was 25 ± 0.5 °C. The DO concentration (C) as a function of time (t) was recorded every 5 s using an optical DO probe (YSI ProODO). Before the addition of the tested compound, the oxygen mass transfer coefficient of the respirometer (k_La) was obtained using a non-steady state procedure (Lobo et al., 2014). When a stable dissolved oxygen concentration (C) was observed, the respirometer was spiked with the tested compound and C as a function of time (t) was recorded. The oxygen uptake rate (OUR) associated with the substrate oxidation (OUR_{Ex}) was calculated from the DO mass balance in the respirometer:

$$OUR_{Ex} = k_La\,(C_e - C) - \frac{dC}{dt} \quad (1)$$

where C_e is the dissolved oxygen concentration in the absence of an oxidizable substrate, and C is the instantaneous dissolved oxygen concentration. In order to compare experiments with different biomass concentrations, the specific exogenous respiration rate (q_{O2}) was calculated as the ratio between OUR_{Ex} and the biomass concentration in the respirometer. Based on the obtained OUR_{Ex} profiles, the oxygen consumed (OC) during the substrate oxidation was calculated as follows:

$$OC = \int_0^t OUR_{Ex}\,dt \quad (2)$$

The oxidation coefficient ($Y_{O/S}$) was calculated as the ratio OC_m/S_0, where OC_m is the maximum amount of oxygen consumed to degrade the added substrate, and S_0 is the initial substrate concentration.

A first set of respirometric assays were carried out to study the effect of the sludge age (SA) on the biodegradation of BPA, 4HAP, 4HB, and 4HBA. In these experiments, additions of 20 mg l^{-1} of the tested compounds were carried out. To analyze the effect of the initial concentration of these compounds on the respiration rate a second set of respirometric assays was performed. Three cycles (C1, C2, C3) of increasing substrate concentration of each compound were carried out; tested concentrations of 4HAP, 4HB, and 4HBA ranged from 10 to 120 mg l^{-1}. Due to the low solubility of BPA, tested concentrations were from 5 to 40 mg l^{-1}. In all the cases, one addition per day was carried out. For all the tested compounds, abiotic experiments (e.g., without biomass) were negative with regard to the oxygen consumption (data not shown).

To evaluate if there were significant differences between the kinetic results obtained with both sludge ages (SA_{30}, SA_{45}) for all the tested compounds, a t-test was performed. Besides, a one-way ANOVA test was used to evaluate if the differences between the stoichiometric parameter ($Y_{O/S}$) corresponding to the three studied conditions (SA_{30}, SA_{45}, initial concentration) were statistically significant (Zar, 1999).

2.5. Analytical techniques

Total suspended solids (TSS) were used to monitor the biomass

concentration (Contreras et al., 2011). Soluble total organic carbon (TOCs) concentration was determined as follows: 5 ml of culture samples were centrifuged for 5 min at 13,000 rpm (Eppendorf 5415C); then, the supernatant was filtered through 0.45 μm cellulosic membranes (Osmonics Inc.). TOCs concentration of the filtered samples was determined in a Shimadzu DOC-Vcpn analyzer. BPA concentration was determined using a colorimetric method (Modaressi et al., 2005). This method uses two reagents, 4-aminoantipyrine (4-AAP) (20.8 mM of 4-AAP in 0.25 M $NaHCO_3$), and ferricyanide (83.4 mM of $K_3Fe(CN)_6$ in 0.25 M $NaHCO_3$) as color generating substrates when combined with phenolic compounds. Colored complexes were measured at 510 nm in a Hach DR 2000 spectrophotometer. Calibration curves were performed periodically using BPA as the reference compound.

3. Results and discussion

3.1. Effect of the sludge age (SA) on the biodegradation kinetics

The effect of the sludge age (SA) on the biodegradation kinetics of the tested compounds was studied. Fig. 1 shows the specific exogenous respiration rate (q_{O2}) as a function of time corresponding to the successive additions of BPA, 4HAP, 4HB, and 4HBA. In general, regardless the sludge age (SA_{30} or SA_{45}) the BPA-acclimated activated sludge was able to degrade all the tested compounds. Fig. 1 shows that the observed maximum q_{O2} value for each pulse was quite sensitive to the noise of the DO signal. Additionally, the maximum q_{O2} value depended on the shape of the respirometric profile of each particular compound. For these reasons, for comparison purposes the specific mean substrate consumption rate (q_{Smean}) was calculated as the ratio between S_o and the total degradation time. According to Lobo et al. (2013), this parameter is a more robust tool to compare the specific degradation activity of compounds with different respirometric profiles as those shown in Fig. 1.

When BPA and 4HAP were tested, the biodegradation time ranged between 5 and 10 h; for this reason, only one addition of substrate per day was possible to carry out (Fig. 1a–d). However, due to the short degradation time of 4HB and 4HBA, up to three additions of these substrates could be performed daily (Fig. 1e–h). Regardless the sludge age, the addition of successive pulses of 4HB and 4HBA caused a gradual increase in the maximum q_{O2} values during each day of the assay. However, it must be noted that maximum q_{O2} values corresponding to the first pulse of each day were similar (Fig. 1e–h). This behavior could be explained considering that the successive additions of the tested compounds caused an increase of the reducing power (e.g., the NADH and NADPH content) of the cells. Because NADH and NADPH are substrates for the monooxygenase responsible for the first oxidation step of 4HBA (Eppink et al., 1997), the increase of reducing power could lead to an enhanced degradation rate. Then, after the last addition of the substrate, the microorganisms consumed the reducing power during the night returning to the basal level. For this reason, maximum q_{O2} values corresponding to the first substrate addition of each day were similar. Therefore, to compare the effect of the sludge age (SA_{30} and SA_{45}) on the biodegradation of 4HB and 4HBA, only the first q_{Smean} value of each day was considered (Table 1). The t-test indicated that there were not significant differences ($T_{(14)} = 0.76$, $p = 0.46$) between the average q_{Smean} values of SA_{30} and SA_{45} when BPA was tested. Conversely, significant differences were obtained between both sludge ages when the analyzed compounds were 4HAP and 4HB (4HAP: $T_{(12)} = 5.71$, $p = 0.000098$; 4HB: $T_{(7)} = 2.94$, $p = 0.02$). In the case of 4HBA, although the q_{Smean} of SA_{45} was lower than that of SA_{30}, the t-test showed that there were not significant differences between the average q_{Smean} values ($T_{(9)} = 0.17$, $p = 0.87$).

3.2. Effect of the substrate concentration

The effect of the substrate concentration on the biodegradation of BPA, 4HAP, 4HB, and 4HBA were analyzed. In these experiments three cycles (named C1, C2, C3) of increasing substrate concentration were carried out. Fig. 2 shows the respirometric profiles corresponding to the three cycles using BPA as substrate. In general, increasing the initial BPA concentration caused an increment of the total degradation time. Considering the same initial concentration, similar q_{Smean} values were obtained in the three tested cycles (C1, C2, C3) (Fig. 6a). For initial BPA concentrations of 5, 10, 20 and 40 mg l^{-1}, average q_{Smean} values were 10.4 ± 1.6, 10.2 ± 3.0, 10.1 ± 0.9, and 12.9 ± 1.0 μmolBPA $gTSS^{-1}$ h^{-1}, respectively. A one-way ANOVA test indicated that there were not significant differences ($F_{(3,11)} = 2.25$, $p = 0.16$) between the average q_{Smean} values corresponding to the tested BPA concentrations.

Figs. 3–5 show the respirometric profiles of 4HAP, 4HB and 4HBA corresponding to the three feeding cycles with increasing substrate concentration. Based on these data, the specific mean substrate consumption rate (q_{Smean}) corresponding to these compounds were obtained (Fig. 6). Respirometric profiles of 4HAP corresponding to the feeding cycles C1, C2, and C3 indicate that maximum q_{O2} values increased as a function of the initial 4HAP concentration (Fig. 3). However, during C1 the extension of the respirometric profiles decreased with the increment of 4HAP concentration. The opposite was observed on the subsequent cycles; during C2 and C3, the extension of the respirometric profiles increased with the increment in the initial 4HAP concentration. These results indicate that during C1 a new acclimation period to 4HAP occurred. The activated sludge used in the respirometric assays was previously acclimated to BPA. Considering that lag phases were not observed during the first feeding cycle (Fig. 3, C1), it can be concluded that the acclimation to BPA conferred to the cells the capability to degrade 4HAP. However, the presence of 4HAP caused a new adjustment in the metabolism of the cells to be more effective to the degradation of this substrate. Considering each 4HAP concentration, the main difference was observed between C1 and the two subsequent feeding cycles (C2, C3); however, q_{Smean} values showed a slight increase from C2 to C3 also (Fig. 6b).

Fig. 4 shows the q_{O2} profiles corresponding to 4HB during the three feeding cycles. During C1 the behavior of q_{O2} was similar to that described for 4HAP; the extension of the respirometric profiles decreased as a function of the increasing 4HB concentrations indicating a new acclimation period. Then, during C2 and C3 the increase in the initial 4HB concentration caused an increment in the extension of the respirometric profiles. Based on these respirometric profiles, q_{Smean} values were obtained. Fig. 6c shows that after the first cycle (C1), q_{Smean} values corresponding to C2 were higher than those obtained during C3, suggesting a loss of biodegradation activity of the biomass with respect to 4HB.

Fig. 5 shows the respirometric profiles obtained by the addition of 4HBA during the three feeding cycles. As it was observed for 4HAP and 4HB, during C1 the extension of the respirometric profiles gradually decreased with the increment in the 4HBA concentration. After C1, the opposite behavior was observed during C2 and C3. Fig. 6d shows that considering each particular initial 4HBA concentration, the major difference with regard to q_{Smean} values was observed between C1 and the next two feeding cycles (Fig. 6d). It must be noted that in general, q_{Smean} values corresponding to C2 were lower than those corresponding to C3. However, during C3

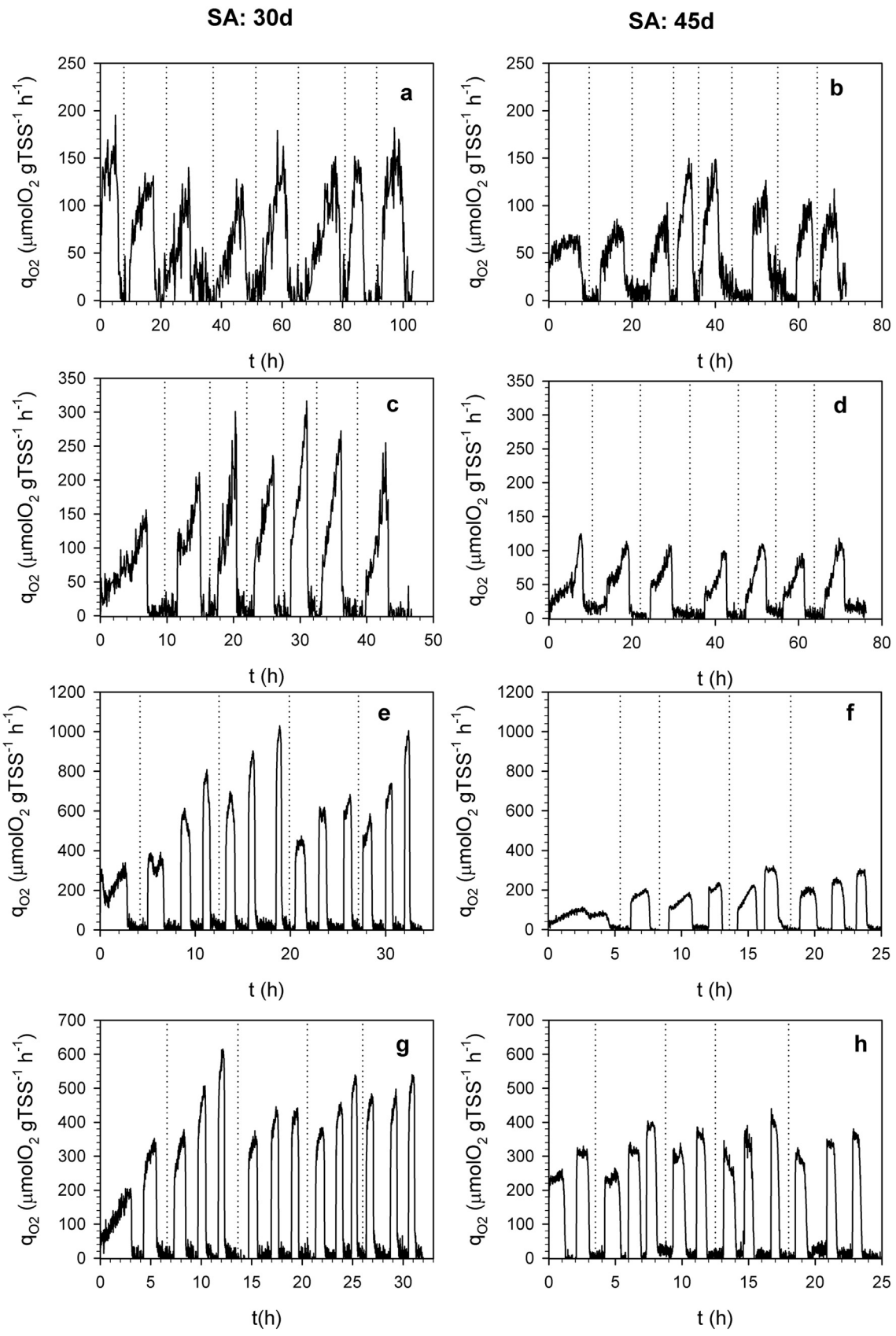

Fig. 1. Effect of the successive substrate additions on the specific oxygen uptake rate (qO_2) of BPA-acclimated activated sludge with sludge age (SA) of 30 and 45 d: (a,b) BPA; (c,d) 4HAP; (e,f) 4HB; (g,h) 4HBA. Dotted lines indicate different days of the assay.

Table 1
Specific mean substrate consumption rate (q_{Smean}) corresponding to activated sludge from SA_{30} and SA_{45} for all the tested compounds. All the values correspond to q_{Smean} ± standard deviation.

Substrate	SA_{30}	SA_{45}
	q_{Smean} (μmolS $gTSS^{-1}$ h^{-1})	
BPA	7.0 ± 1.9	7.7 ± 1.7
4HAP	26.2 ± 2.9	10.6 ± 0.4
4HB	101.4 ± 23.5	39.3 ± 19.7
4HBA	106.3 ± 25.0	91.0 ± 14.6

and for 4HB concentrations higher than 60 mg l^{-1}, a gradual decrease of q_{Smean} values were observed. Finally, when 120 mg 4HBA l^{-1} was tested, q_{Smean} value corresponding to C3 was lower than the value obtained during C2 (Fig. 6d).

The results described in this section suggest that the kinetics of the biodegradation of the tested compounds was influenced by many factors, such as the sludge age, the acclimation process, and the concentration of the analyzed compound. Although q_{Smean} values of a particular compound depends on the above mentioned factors, obtained results show that as a general rule, the tested compounds can be ordered according to their biodegradation rates as follows: BPA < 4HAP << 4HB ≤ 4HBA. Therefore, the BPA oxidation rate limits the rate of the whole biodegradation pathway and the accumulation of its metabolic intermediates 4HAP, 4HB, and 4HBA is negligible.

3.3. Stoichiometry of the aerobic biodegradation of BPA, 4HAP, 4HB and 4HBA

Based on the respirometric profiles shown in Figs. 1–5, the oxidation coefficients ($Y_{O/S}$, $molO_2$ $molS^{-1}$) were calculated as the ratio between the maximum oxygen consumed (OC_m) and the initial substrate concentration (S_o). Considering the different tested sludge ages (SA_{30} and SA_{45}), the acclimation degree and the initial concentration (cycles C1, C2, C3), Table 2 shows that average values of $Y_{O/S}$ corresponding to BPA, 4HAP, 4HB, and 4HBA were quite constant. For each tested compound, a one-way ANOVA test showed that no differences between the three evaluated conditions were found (BPA: $F_{(2,25)} = 2.7$, $p = 0.08$; 4HAP: $F_{(2,17)} = 2.11$, $p = 0.15$; 4HB: $F_{(2,47)} = 1.58$, $p = 0.22$; 4HBA: $F_{(2,34)} = 1.17$, $p = 0.32$).

For all the studied compounds, $Y_{O/S}$ values obtained in the present work were lower than those corresponding to the complete oxidation of the compound (Table 2). This result clearly indicates that none of these compounds was completely oxidized (e.g., mineralized) to CO_2. Moreover, from the ratio between the experimental $Y_{O/S}$ values and those corresponding to the complete oxidation of each compound, it can be concluded that about 40–60% of the available electrons of the tested compounds were transferred to the biomass. Considering BPA, the result was in agreement with that reported in a previous work (Ferro Orozco et al., 2015).

The $Y_{O/S}$ values shown in Table 2 are in accordance with the aerobic biodegradation pathway of BPA and its metabolic products

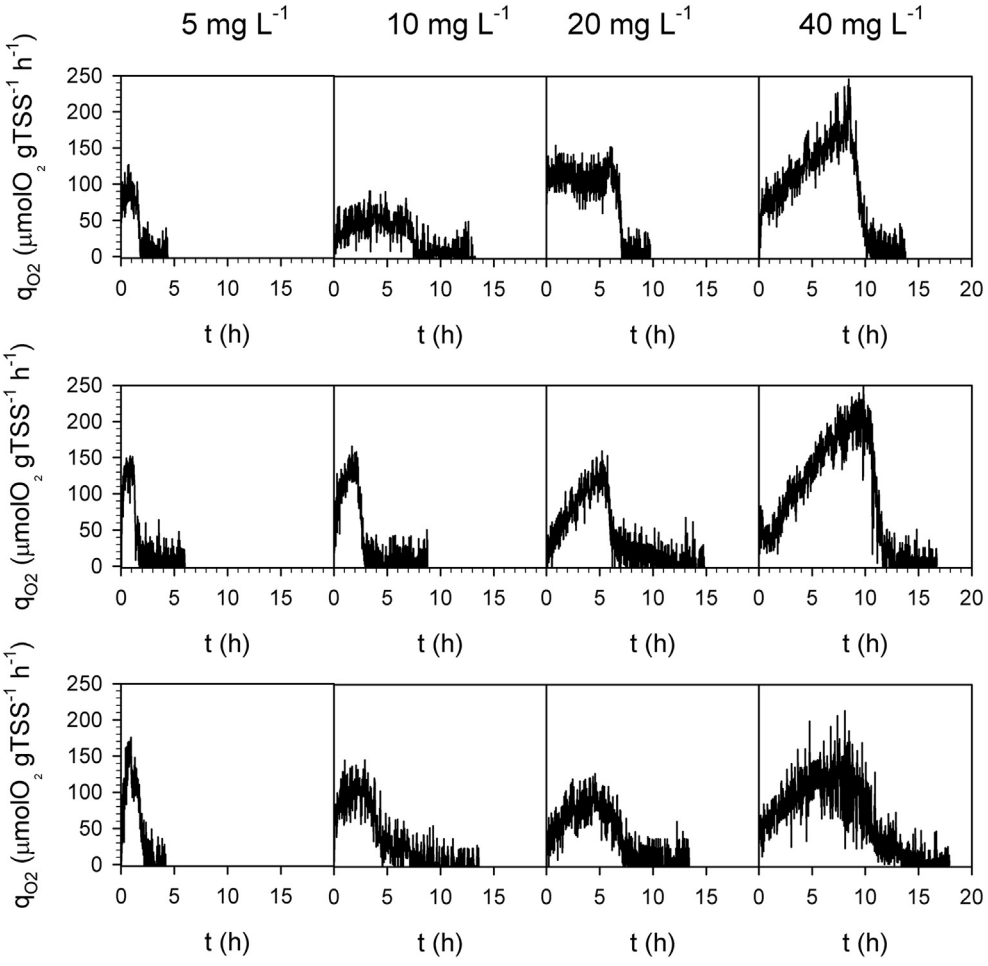

Fig. 2. Specific oxygen uptake rate (qO_2) as a function of time corresponding to increasing BPA concentrations during three feeding cycles (C1, C2, C3).

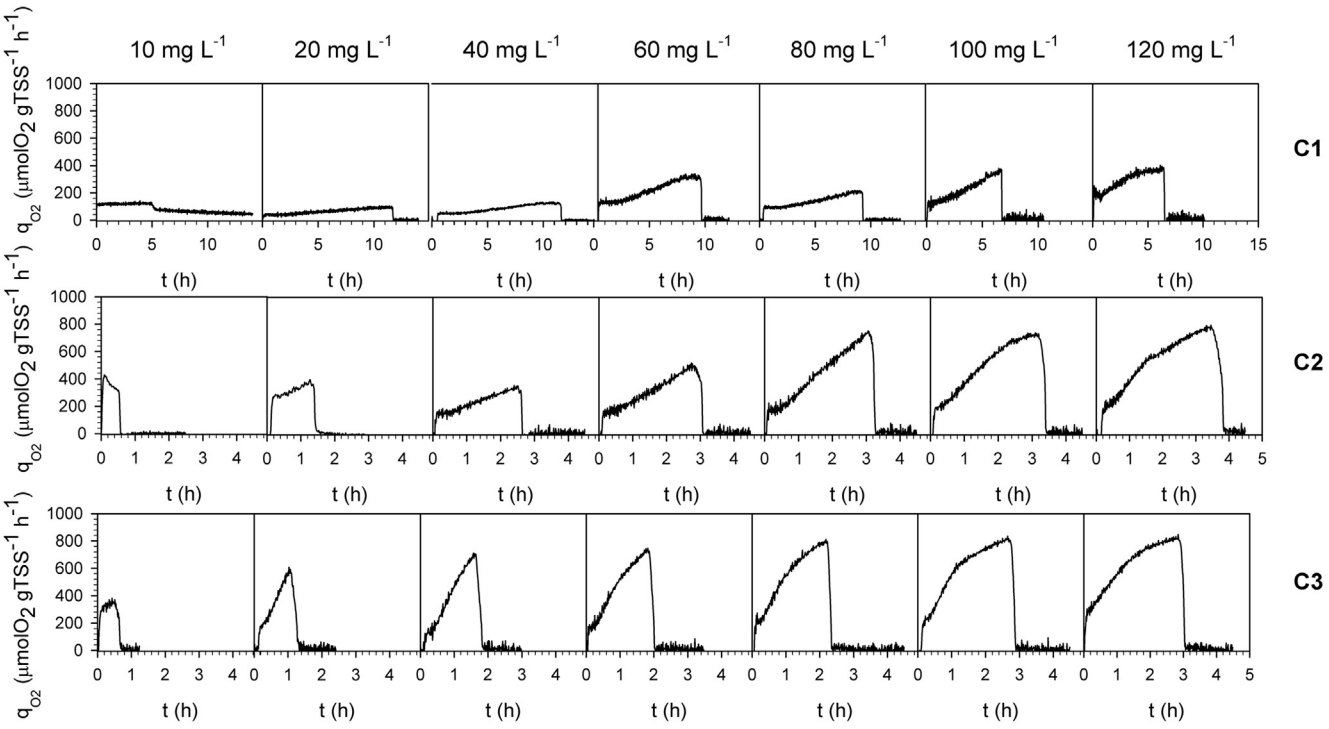

Fig. 3. Specific oxygen uptake rate (qO_2) as a function of time corresponding to increasing 4HAP concentrations during three feeding cycles (C1, C2, C3).

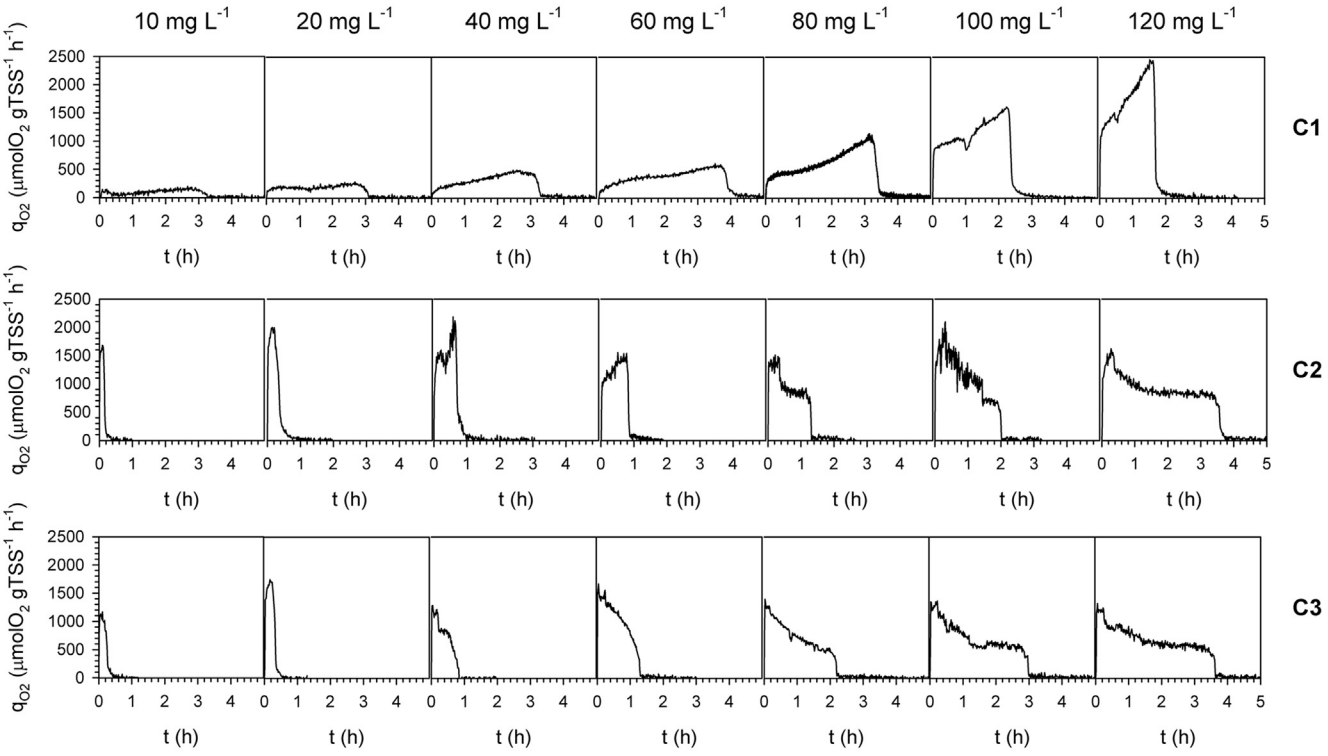

Fig. 4. Specific oxygen uptake rate (qO_2) as a function of time corresponding to increasing 4HB concentrations during three feeding cycles (C1, C2, C3).

reported by Lobos et al. (1992), Spivack et al. (1994), Sakai et al. (2007) and Fischer et al. (2010). According to those authors, the main aerobic pathway for the primary metabolization of BPA ($C_{15}H_{16}O_2$) comprises the sequential action of a monooxygenase, a dehydratase and a dioxygenase to form the 4HB ($C_7H_6O_2$) and 4HAP ($C_8H_8O_2$). The overall stoichiometry of this step is as follows:

$$C_{15}H_{16}O_2 + 2O_2 + NADH + H^+ \rightarrow NAD^+ + 2H_2O + C_7H_6O_2 + C_8H_8O_2 \quad (3)$$

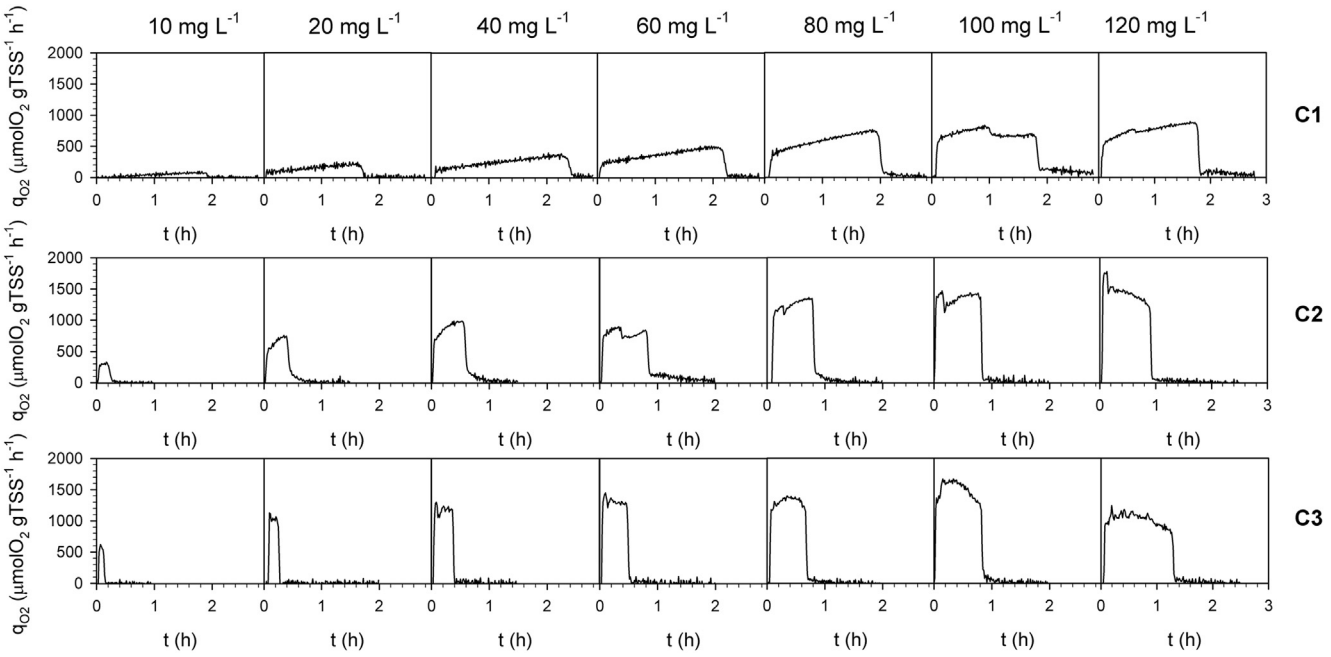

Fig. 5. Specific oxygen uptake rate (qO_2) as a function of time increasing 4HBA concentrations during three feeding cycles (C1, C2, C3).

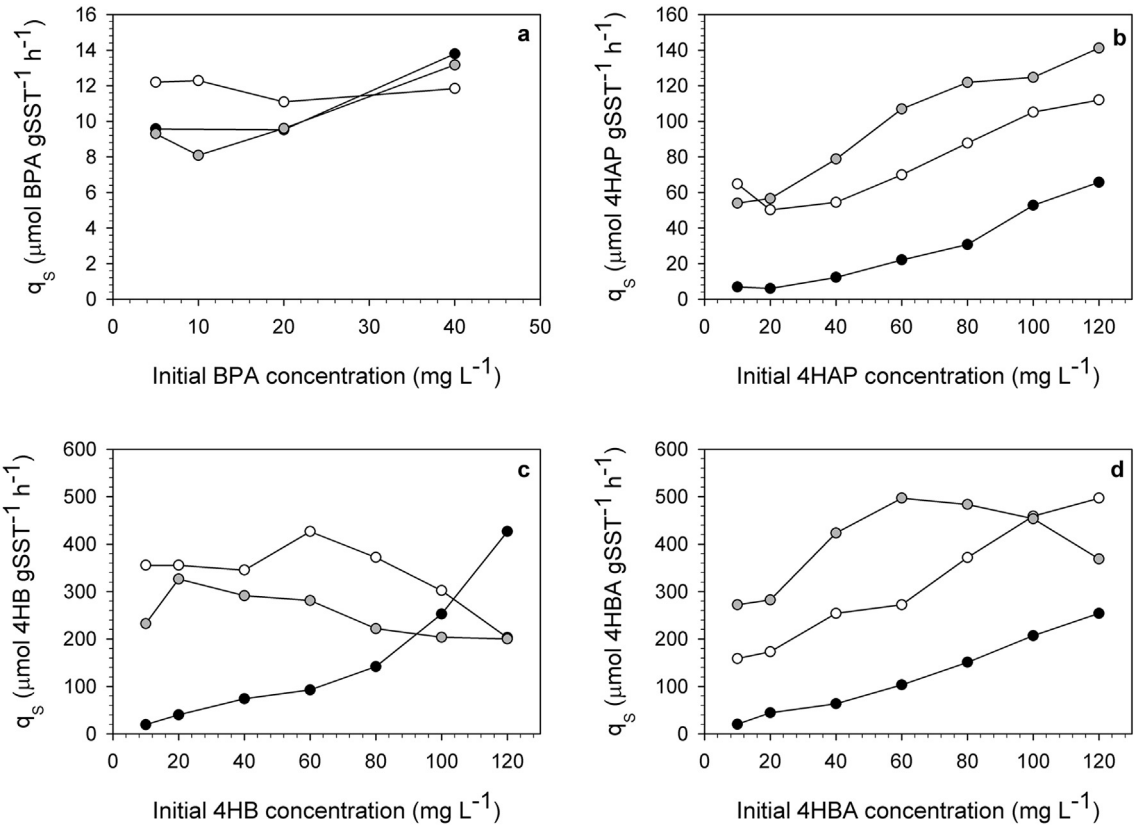

Fig. 6. Specific mean substrate consumption rate (qSmean) as a function of the initial concentration of the tested substrates. (a) BPA, (b) 4HAP, (c) 4HB, (d) 4HBA. Different colors of the symbols indicate different feeding cycles: (●) C1, (○) C2, (●) C3.

4HB is oxidized to 4HBA ($C_7H_6O_3$) by a dehydrogenase, producing 1 mol of NADH (Spivack et al., 1994). Taking into account that in this work respirometric experiments were performed under starvation conditions (e.g., one to three additions of a single substrate with a low initial concentration per day), the NADH generated from the oxidation of 4HB could be readily oxidized by oxygen to generate energy via oxidative phosphorylation. Under these conditions, the oxidation of 4HB to 4HBA is coupled with the

Table 2
Comparison between the oxidation coefficient ($Y_{O/S}$) corresponding to the complete oxidation, the experimental values and the obtained from the aerobic biodegradation pathway (Eqs. (3)–(11)) for the tested compounds.

$Y_{O/S}$ ($molO_2\ molS^{-1}$)	BPA	4HAP	4HB	4HBA
Complete oxidation	18	9	7.5	7
SA_{30} (20 mg L^{-1})	9.8 ± 1.9	5.7 ± 0.5	3.9 ± 0.4	3.5 ± 0.4
SA_{45} (20 mg L^{-1})	9.2 ± 1.4	5.4 ± 0.2	3.9 ± 0.6	3.0 ± 0.4
SA_{30} (C1–C3)	11.0 ± 2.0	4.9 ± 0.5	3.6 ± 0.6	2.9 ± 0.4
Experimental value	10.0 ± 0.5	5.2 ± 0.2	3.7 ± 0.2	3.0 ± 0.1
Proposed pathway	10.5	5.0	3.5	3.0

consumption of one-half mole of molecular oxygen:

$$C_7H_6O_2 + 1/2\ O_2 \rightarrow C_7H_6O_3 \quad (4)$$

Early studies demonstrated that 4HBA ($C_7H_6O_3$) can be oxidized by 4-hydroxybenozate 3-monooxygenase to produce 3,4-dihydroxybenzoate ($C_7H_6O_4$) (Fujii and Kaneda, 1985). This compound can be further oxidized by several dioxygenases to form different open ring intermediates (ORI). Then, these ORI enter the intermediary metabolism to serve as carbon and energy source for the microbial growth. Although several metabolization routes of these ORI are reported, in all cases beyond this point oxygenases are absent (for more details, see Gao et al., 2010). The overall oxidation of 4HBA ($C_7H_6O_3$) to ORI can be represented as follows:

$$C_7H_6O_3 + NADPH + H^+ + 2O_2 \rightarrow NADP^+ + H_2O + ORI \quad (5)$$

More recently, Eppink et al. (1997) showed that 4HBA ($C_7H_6O_3$) can also be oxidized by 4-hydroxybenzoate 1-hydroxylase to produce hydroquinone ($C_6H_6O_2$):

$$C_7H_6O_3 + NADPH + H^+ + O_2 \rightarrow NADP^+ + H_2O + CO_2 + C_6H_6O_2 \quad (6)$$

Hydroquinone (HQ) can be oxidized by phenol 2-monooxygenase to benzenetriol (Eppink et al., 2000) and then to maleylacetic acid ($C_6H_6O_5$) by hydroxyquinol 1,2-dioxygenase (Jain et al., 1994):

$$C_6H_6O_2 + NADPH + H^+ + 2O_2 \rightarrow NADP^+ + H_2O + C_6H_6O_5 \quad (7)$$

Finally, maleylacetic acid enters the intermediary metabolism. More information concerning the metabolization of maleylacetic acid can be found in Gao et al. (2010). In a previous work, Lobo et al. (2013) studied the oxidation of several phenolic compounds by phenol-acclimated activated sludge; those researchers reported a $Y_{O/S}$ of 2 mol O_2 per mol of HQ oxidized, which is in accordance with Eq. (7).

Taking into account Eqs. (6) and (7), the overall oxidation of 4HBA ($C_7H_6O_3$) to CO_2 and maleylacetic acid ($C_6H_6O_5$) is:

$$C_7H_6O_3 + 2NADPH + 2H^+ + 3O_2 \rightarrow CO_2 + 2NADP^+ + 2H_2O + C_6H_6O_5 \quad (8)$$

Eqs. (5) and (8) demonstrate that 2–3 mol of oxygen are consumed per mole of 4HBA oxidized due to the action of oxygenases. Note that the latter value is in accordance with the experimental $Y_{O/S}$ value corresponding to 4HBA obtained in the present work (Table 2). Moreover, from the combination of Eqs. (4) and (8) it can be concluded that $Y_{O/S}$ value corresponding to 4HB would be 3.5 $molO_2\ molS^{-1}$, which is close to the experimental one (Table 2).

The 4HAP ($C_8H_8O_2$) formed during the cleavage of BPA (Eq. (3)) can be oxidized to 4-hydroxyphenyl acetate by 4-hydroxy acetophenone monooxygenase, and then, it is cleaved to HQ ($C_6H_6O_2$) and acetic acid ($C_2H_4O_2$) (Tanner and Hopper, 2000):

$$C_8H_8O_2 + NADPH + H^+ + O_2 \rightarrow NADP^+ + C_2H_4O_2 + C_6H_6O_2 \quad (9)$$

Then, HQ is metabolized to maleylacetic acid (MAA) via Eq. (7). Depending on the environmental conditions, acetate could be accumulated, stored as PHB (e.g., under the excess of carbon and energy source), or oxidized to CO_2 (e.g., under famine conditions). In the present work, the starvation conditions employed in the assays could favor the oxidation of acetate to obtain energy. In this sense, it must be pointed out that Ferro Orozco et al. (2013) reported that the oxidation rate of acetate by acclimated BPA-activated sludge was about two fold the values of the non-acclimated ones. Assuming a complete oxidation of acetate and combining Eqs. (7) and (9), the overall oxidation of 4HAP ($C_8H_8O_2$) to MAA ($C_6H_6O_5$) and CO_2 is:

$$C_8H_8O_2 + 2NADPH + 2H^+ + 5O_2 \rightarrow 2NADP^+ + 2CO_2 + 3H_2O + C_6H_6O_5 \quad (10)$$

According to Eqs. (10) and (5) mole of oxygen are consumed during the oxidation of 4HAP to MAA; this value is close to the $Y_{O/S}$ value corresponding to 4HAP obtained in this work (Table 2).

The overall oxidation of BPA to MAA can be obtained from the combination of Eqs. (3), (4), (8) and (10):

$$C_{15}H_{16}O_2 + 10.5\ O_2 + 5NAD(P)H + 5H^+ \rightarrow 5NAD(P)^+ + 7H_2O + 3CO_2 + 2C_6H_6O_5 \quad (11)$$

According to Eq. (11), 10.5 mole of O_2 are consumed per mol of BPA degraded. To obtain Eq. (11) it was assumed that the experiments were performed under starvation conditions. If this is the case, the NADH produced during the oxidation of 4HB to 4HBA is readily oxidized by oxygen (Eq. (4)) and that the acetate produced in the cleavage of 4HAP is completely oxidized to obtain energy (Eq. (10)). It must be noted that the agreement between the $Y_{O/S}$ corresponding to BPA (Eq. (11)) and the experimental value (Table 2) supports the above mentioned assumptions.

Results reported in the present work indicate that even when the biodegradation kinetics of the tested compounds was strongly affected by many factors, such as sludge age, acclimation degree and compound concentration, the stoichiometry of the biodegradation reactions remained quite constant regardless the experimental conditions.

4. Conclusions

A respirometric technique was used to study the kinetics and stoichiometric characteristics of the aerobic degradation of BPA and its metabolic intermediates by activated sludge. The effect of the sludge age, and the concentration of each tested compound was analyzed.

Obtained results indicate that kinetics of the biodegradation of the tested compounds was influenced by the sludge age, the acclimation process, and the concentration of the analyzed compound. In the case of BPA and 4HBA, q_{Smean} values for both SA_{30} and SA_{45} were similar. Conversely, when 4HAP and 4HB were tested, the biomass of SA_{30} had a higher specific respiration activity than SA_{45}. Within the tested initial BPA concentrations, q_{Smean} values were approximately constant. However, considering 4HAP, 4HB, and

4HBA, results show that during the first feeding cycle (C1) a new acclimation period occurred. The tested compounds can be ordered according to their biodegradation rates as follows: BPA << 4HAP < 4HB ≤ 4HBA. These results indicate that the oxidation rate of BPA limits the rate of the whole biodegradation pathway and that the accumulation of its metabolic intermediates 4HAP, 4HB, and 4HBA is negligible.

The stoichiometry of the biodegradation reactions remained constant regardless the experimental conditions. $Y_{O/S}$ values obtained in the present work were lower than those corresponding to the complete oxidation of each tested compound. Thus, none of the compounds was completely oxidized (mineralized) to CO_2. From the comparison between the metabolic pathways of BPA reported in literature and $Y_{O/S}$ values obtained in the present work corresponding to each tested compound, the aerobic biodegradation pathway of BPA by BPA-acclimated activated sludge was discussed.

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